

CRISPR-CAS9 fighting human immunodeficiency virus HIV-1 subtype in CD4+ T lymphocytes: a literature review**CRISPR-CAS9 e combate ao vírus da imunodeficiência humana subtipo HIV-1 em LINFÓCITOS T CD4+: uma revisão de literatura**

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ABSTRACT

The human immunodeficiency virus (HIV) requires glycoproteins and specific receptors found in the host and its immune system, like so glycoprotein 120 is responsible for binding to the CD4+ molecule and later binding to the CCR5 or CXCR4 co-receptors. Based on these mechanisms, cell entrance can occur for the replication of viral genetic material. After various investigations on the way bacteria act when facing viral invaders, the CRISPR-Cas9 tool was an explicit protection promoter against HIV-1 in humans. Currently, studies about the simultaneous knockout of CCR5 and CXCR4 genes in CD4+ T cells via CRISPR-Cas9 confer resistance to HIV infection. In this context, research related to the CCR5 delta 32 mutation has a high degree defense against HIV. Besides, mutations in co-receptors may explain the lack of infections in this group. Lastly, a CRISPR-Cas9 technique represents a major breakthrough against HIV-1 infection from co-receptor issues, making it impossible for the virus to attach the cell. From this review, it was possible to observe the importance of the genetic engineering tool CRISPR-Cas9 to be used as a way to treat people affected with HIV, through approaches in CCR5 and CXCR4 co-receptors, as well as alternative methods for its use when the virus is at intracellular latent state.

Keywords: *CRISPR-Cas9; HIV-1; CCR5; CXCR4; co-receptors; HIV.*

RESUMO

O vírus da imunodeficiência humana (HIV) necessita de glicoproteínas e receptores específicos no hospedeiro e em seu sistema imunológico, dessa forma a glicoproteína 120 é responsável pela ligação à molécula CD4+ e posterior ligação aos co-receptores *CCR5* ou *CXCR4*. Com base nestes mecanismos, pode ocorrer entrada na célula para a replicação do material viral. Após diferentes estudos sobre a forma de atuação das bactérias contra invasores virais, a ferramenta *CRISPR-Cas9* tem sido elucidada para promover mecanismos de proteção contra o HIV-1 em seres humanos. Atualmente, estudos apontam que o *knockout* simultâneo de genes *CCR5* e *CXCR4* em células T CD4+ via *CRISPR-Cas9* confere resistência à infecção pelo HIV. Neste contexto, pesquisas relatam que indivíduos com a mutação *CCR5* delta 32 apresentam alto grau de proteção à infecção pelo HIV. Sendo assim, mutações em co-receptores poderiam explicar a falta de infecções nesse grupo. Por fim, a técnica *CRISPR-Cas9* representa um grande avanço contra infecção por HIV-1 a partir de edições nos co-receptores, impossibilitando a ligação do vírus à célula. A partir dessa revisão foi possível observar a importância da ferramenta de engenharia genética *CRISPR-Cas9* para ser utilizada como uma forma de tratar pessoas acometidas com o vírus da imunodeficiência humana através de abordagens nos co-receptores *CCR5* e *CXCR4*, além de métodos alternativos para utilização quando o vírus se encontra de forma latente na célula.

Palavras-chave: *CRISPR-Cas9; HIV-1; CCR5; CXCR4; co-receptores; HIV.*

1 INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) affects over 37 million people worldwide, with approximately 2 million people infected each year. Treatment with antiretroviral therapy (ART) aims to inhibit active viral replication to bring viral loads to undetectable levels. ART has advanced in the clinical management of HIV-1 infection, but

so far the cure is not considered because the virus develops at residual level, known as a latent HIV reservoir [1].

There are two types of HIV, the M-tropic type, which mainly affects macrophages infecting T cells through CCR5 co-receptors, and T-tropic, which preferentially go for T lymphocytes from CXCR4 co-receptors. The M-tropic chains participate in the initial infection phase. When the targets of HIV are macrophages, their function is to phagocytose the chains attempting to process proteins for later cell presentation to the immune system. T-tropic viruses induce the processing and destruction of T-cell populations that lead to the illness (AIDS). The CD4⁺ T lymphocytes that have the CXCR4 co-receptor become infected in nodules and other lymphatic tissues. That way, HIV keeps stabilized and it's continuously presented to lymphatic tissue because it's where T lymphocyte proliferation occurs, attempting to limit infection and destroy the host-virus [2].

Chemokines receptors act as co-receptors along with CD4⁺, for cell invasion and the onset of the HIV replicative cycle [3]. From this interaction, subsequent changes occur resulting in the exposure of the CCR5 or CXCR4 chemokines co-receptor molecules, relevant in HIV infection. While CCR5 is used to interact with true variants in the early and asymptomatic phase of infection. As a greater ability to escape the immune system is suggested, CXCR4 has a special role in the advanced phase of infection and therefore its use by the virus represents greater CD4⁺ T lymphocyte depletion and faster immunodeficiency development [4].

The CRISPR-Cas9 system might eradicate HIV infection, knowing that several promising approaches have been established by the CRISPR-Cas9 system. One such approach is the editing of CCR5 or CXCR4 co-receptors. These co-receptors are essential for virus access, so defects around these will interfere or block HIV infection. Several reports have successfully demonstrated mutation of the CCR5 or CXCR4 gene, targeting these genes that subsequently leads to the safeguard of primary CD4⁺ T cells from HIV-1 [3].

To better understand the mechanisms related to genetic editing, this study aims to provide information on the main strategies associated with the functional cure of HIV-1 infection by editing the CXCR4 and CCR5 genome through CRISPR-Cas9 [5].

2 MAIN TEXT – REVIEW – LITERATURE REVIEW**2.1 HUMAN IMMUNODEFICIENCY VIRUS**

The human immunodeficiency virus contamination is caused by blood or sexual contact, the virus initially adheres to dendritic cells and macrophages. When attached, the virus is transported to the lymph nodes where they are stored and defense cells are produced. Because viruses are obligate intracellular parasites, to stay alive they act in the primary hours after infection. This occurs when the viral envelope glycoprotein complex, gp120, and gp41 specifically connects to the host cell with the CD4+ molecule and CXCR4 or CCR5 chemokine co-receptors [6].

When HIV attaches to the target cell, the T lymphocyte, and its outer envelope is discarded. Thusly, the capsid that is the virus's content penetrates the cytoplasm and is digested by the cell. Following capsid breakdown, the main components of HIV: RNA, reverse transcriptase, protease, and integrase are released within the host cell. Starting the process, reverse transcriptase begins its function by translating cellular DNA, transforming viral RNA into viral DNA. From the formation of the double strand of viral DNA, the integration to the host cell genetic material occurs with the aid of the integrase enzyme. The T lymphocyte after being infected by the virus initiates the production of viral proteins and the formation of new viral RNAs. At this moment, there is the action of the protease enzyme, which cleaves the precursor viral protein into smaller and mature proteins. After that, RNA and proteins are released to infect other cells [4].

Due to HIV infection, 5% of CD4+ lymphocytes infected suffer apoptosis after a period of viral replication that occurs after the binding of viral DNA to CD4 lymphocyte DNA, with viral DNA being produced from cellular RNA itself, triggering virus production and causing apoptosis to the infected cell. However, 95% of CD4+ lymphocytes suffer from pyroptosis, which is a type of inflammatory cell death after failed attempts by the virus to complete its cycle. Pyroptosis happens in cases where replication is not completed and remnants of viral DNA persist in the cell. These viral fragments cause an inflammatory reaction leading to cell death. This process causes the spread of its cytoplasmic content with pro-inflammatory substances, attracting new CD4+ and restarting the chain. The CD4+ cells have a defense mechanism that prevents the virus from completing the production of DNA from its RNA by reverse transcription, this way, accumulating viral particles that will not complete the replication cycle and are degraded by inflammatory response [7].

3 CRISPR-CAS9 GENETIC TOOL

This tool consists of bacterial defense against viral attack. When bacteria detect the presence of viral DNA, two types of short RNAs are produced where one contains the invading virus sequence. These RNAs form a complex along a protein called Cas9. Cas9 is a nuclease that can cleave DNA when a known sequence (guide RNA) composed of 20 base pairs bind to the 20 base pairs from the region of interest finding its target in the viral genome. Then Cas9 cleaves its target by disabling the virus. This mechanism can be engineered to reduce not only viral DNA but any DNA sequence at the chosen site [8].

The CRISPR-Cas9 system is categorized into three types, all of which type II is the most used because it is composed of multifunctional proteins such as Cas9. The Cas genes produce a type of protein that cooperates in the molecular memory of bacteria. The Cas9 protein is formed from bacterial strains, derived from the bacteria *Staphylococcus aureus* (SaCas9) or *Streptococcus pyogenes* (SpCas9), that can section DNA at any genomic locus of interest [9].

Despite similarities, there is a great abundance in CRISPR-Cas9 systems found in different bacteria. The CRISPR-Cas9 type II system derived from the bacterium SpCas9 is the most studied due to its lower complexity and easier application in gene therapy [8].

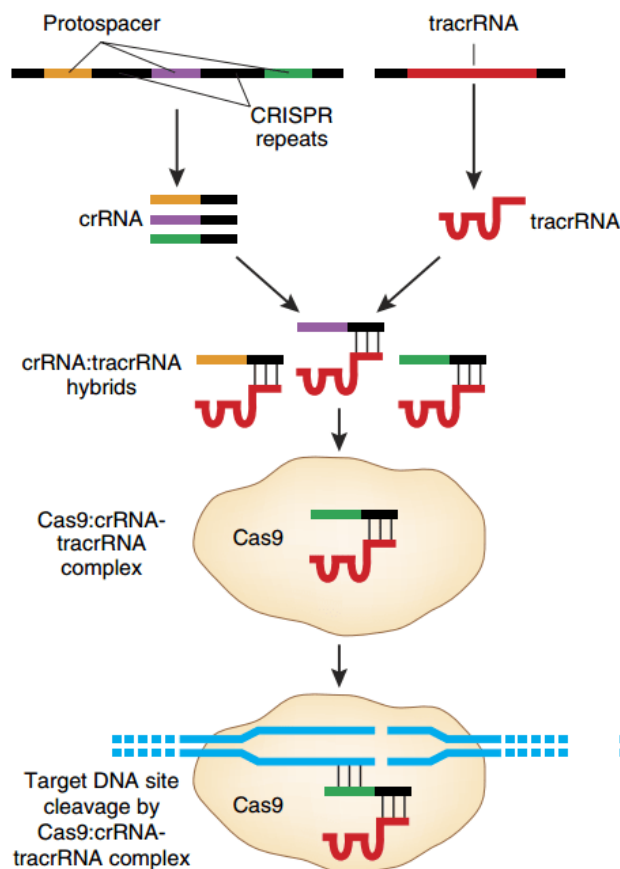
In the CRISPR-Cas9 system, several target genetic sites can be directed simultaneously using multiple guide RNA (gRNA) sequences. In other genetic techniques such as TALENs and ZFNs, there is a need for laboratory procedures of protein design and resynthesis, that may lead nucleases to the chosen site, while Cas9 nuclease redirection depends on two RNA sequences. Cas9 nuclease is driven by an RNA sequence that recognizes target DNA by complementarity, thereby making it accessible to Cas9 action. Thus and so, the various advantages of the CRISPR method are clear compared to other gene editing techniques [10].

Structurally Cas9 contains 2 independent enzymatic properties, HNH and RuvC, names borrowed from homology to the corresponding endonucleases. Each property is responsible for the cutting a DNA strand [11]. Structurally less complex, HNH cleaves the complementary DNA strand, while more complex RuvC cleaves the non-complementary DNA strand. Recently, based on a structural analysis of CRISPR-Cas9 it was possible to verify the existence of two lobes with distinct functions, one of recognition (REC) and one of nuclease (NUC). In REC, Cas9 has interaction with RNA-DNA in several ways, allowing

to understand that Cas9 nuclease has no specificity in targeting, so the attendance of crRNA-tracrRNA is essential for its activity [12].

In spite of that, even with the crRNA:tracrRNA duplex form the Cas9 cut process is not completely specific so, off-target cleavage and off-target genome editing might occur. This type of editing refers to unspecific and unintended genetic alterations. Accordingly, guide RNA (gRNA) is the fusion between crRNA and tracrRNA that recruits Cas9, thereby directing the Cas9 nuclease to the genomic target near the *Protospacer Adjacent Motif* (PAM) sequence. The PAM sequence, is a guanine-guanine nucleotide sequence, and it may be found at various points in the genome, allowing the technique to be applied in different places of interest. Cleavage of the genomic target recruits the repair mechanism by the junction of non-homologous tips, making it easy to insert *indels*, a term in molecular biology for the insertion or suppression of bases in the genome of an organism [13,14].

Figure 1. Protospacer-derived crRNA hybridizes to tracrRNA. The hybrid formed a complex with Cas9, leading to the target DNA where the protospacer region is linked by complementarity, allowing the cleavage by Cas9.

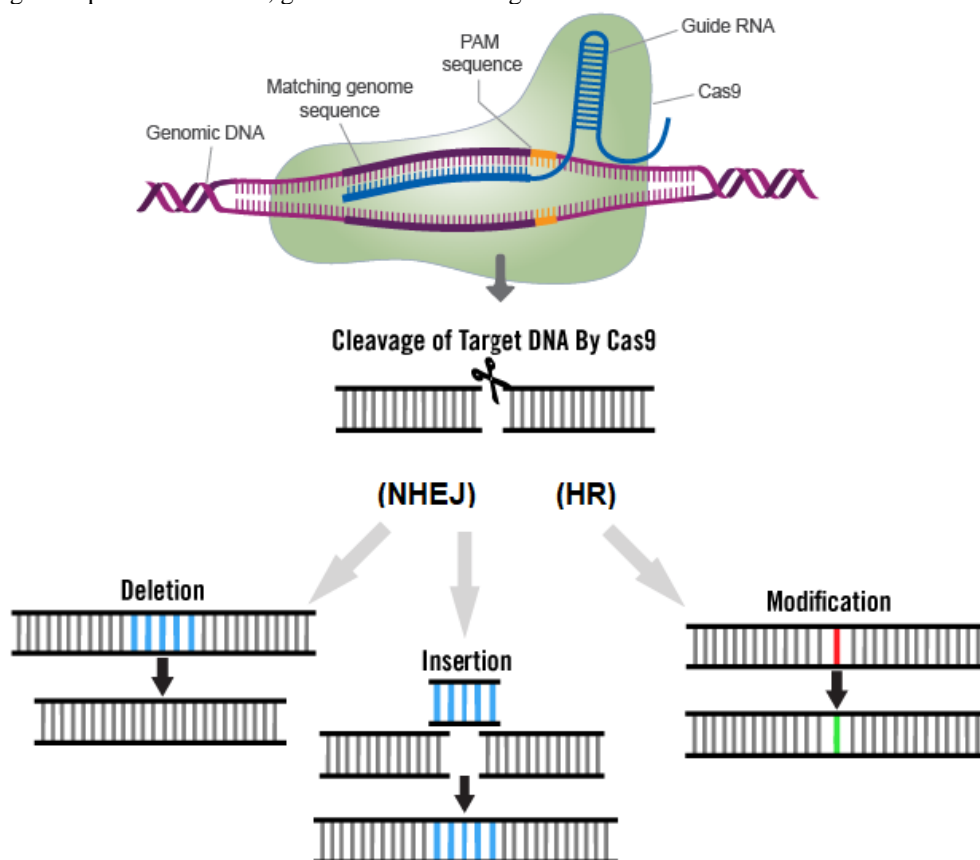


Source: (Pečnerová P.C. (2016) “The almighty CRISPR-Cas9 technology: How does it work?”.)

4 REPAIR MECHANISM POST CLEAVAGE

Double strand cleavage will be processed by 2 DNA repair mechanisms, the homologous recombination (HR) being a precise system using the corrective synthetic sister chromatid DNA strand to repair, reducing the error through that template to the formation of DNA repairing in a homologous way. Non-homologous recombination (NHEJ) is more active than HR because it does not require a DNA template, but is more prone to errors through small insertions or genetic deletions. These mutations may lead to alteration of target gene function [10].

Figure 2. NHEJ: Non-homologous repair mechanism, gene changes by small insertions or deletions. HR: Homologous repair mechanisms, gene correction through a model.



Source: (modified from Cravero M. (2016) "Embrione: scienza e riproduzione." And, Bio M. (2018) "CRISPR/Cas9 Genome Editing: Transfection Methods.")

5 TRANSPORT FROM CRISPR / CAS9 TO CELLS

5.1 PLASMIDS

Plasmids provide the best-known method for transporting the CRISPR-Cas9 system to target cells with effortless in vitro production as advantage. Cells are simultaneously transfected with plasmids encoding Cas9, crRNA, and tracrRNA by electroporation

methods [11]. However, the applicability of this procedure to humans will only be in ex vivo cases. Simplification forms of this method have been developed by creating a plasmid encoding sgRNA and, recently a vector encoding on the same plasmid Cas9 and sgRNA, reducing the number of plasmids formed from three to one. This technology allows the plasmids to be rearranged to encode multiple gRNAs, allowing multiple-locus corrections [15].

In vivo studies shows some limitations of this method for the sake of its low efficiency and epigenetic silencing. It has been observed that in a proportion of transfected cells, there was random integration of genetic material into the host genome, allowing continuous production of Cas9 and gRNA. Although, may trigger off-target effects and insertion mutations [14,16].

5.2 VIRAL VECTORS

Viral vectors are used as a mechanism for introducing an exogenous DNA fragment into primary cells or cells refractory to plasmid transfection [14].

Lentiviruses can randomly integrate an exogenous DNA fragment into the target cell's genetic material and can generate unwanted insertions into the host locus. Therefore, it constitutes a limitation to the use of this type of viral vectors [17].

To avoid this problem, alternatives such as non-integrative viral vectors like adenoviruses and adeno-associated viruses (AAVs) [11] are used, for viral DNA disappears after some mitotic cycles [18]. Their episomal nature has the great ability to be cloned and transduced in numerous cell lines, and their ease of production increases as result of their potential use. Vitro studies show that adenoviruses have a good correlation between virus size and packaging efficiency, but when used in vivo they can induce host immune response, with increased inflammatory cytokines and may lead to cell destruction [19,20]. The AAVs, on the other hand, induce an accelerated immune response and can provide lasting expression in cells that are not dividing. The smaller size of this type of virus raises concerns about Cas9 gene packaging. However, this problem can be solved by using a Cas9 from SaCas9 bacteria although small, it demonstrated to have the same efficiency as SpCas9 Cas9, without increasing the number of mutations off-target [19].

5.3 AN IN VIVO AND EX VIVO APPROACH

The CRISPR-Cas9 method can be applied by modifying human cells in culture, then transplanted to humans or potentially in vivo by editing directly to target cells in the interested organism [2].

6 CRISPR-CAS9 ACTING ON HIV-1 CO-RECEPTORS

The CRISPR-Cas9 engine has several approaches to act against HIV-1, one of them directly in the co-receptors [21].

Studies related to 'knockout' that is used to inactivate or block the expression of a specific gene in an organism, became possible after reports that individuals with a 32 base pair homozygous deletion of the CCR5 gene also known as CCR5-delta-32, were healthy and resisted HIV-1 infection [22].

HIV-1 penetrates host cells by binding to the CD4+ receptor and the CXCR4 or CCR5 co-receptors. Knowing that the CD4+ antigen is indispensable for the functional immune system, being not possible to discontinue it as a strategy to prevent HIV-1 infection. In consequence, CCR5 and CXCR4 co-receptors become potential targets in this HIV-1 gene therapy.

The use of adenovirus-supplied CRISPR-Cas9 technique combined with sgRNAs has the objective to prevent CCR5 expression. This can induce disruption of this co-receptor in TZM-BL cells, an HIV-sensitive cell type. Thus, using this technique in human CD4+ T cells, it is possible to silence CCR5 expression resulting in cellular protection [23].

The administration of CRISPR-Cas9 technique on pluripotent hematopoietic stem cells with CD34 antigens, made possible to rupture CCR5 co-receptor in vitro, causing the inhibition of HIV-1 infection [10].

Regardless of that, the side effects of permanent editing of the CCR5 gene have not been reported, although some research shows that CCR5 deficiency increases the risk of symptomatic West Nile virus infection.

CRISPR-Cas9 disruption of the CXCR4 co-receptor was efficient in CD4+ T cells using lentiviruses and two sgRNAs that specifically target conserved CXCR4 sequences. The reduction of p24 nucleocapsid can be observed in CD4+ T cells after its knockout, that demonstrates efficiency in co-receptor disruption [24].

Was also possible to discontinue CXCR4 expression by Cas9 and gRNA in human CD4+ T cells, it reduced about 40% of expression at the CXCR4 cell surface [25]. Also, a

SaCas9, that is 1 kb shorter than SpCas9, was used to suspend CXCR4 in adeno-associated virus-delivered human primary CD4+ T cells and protected them from HIV-1 infection with little out-of-range effects target and low toxicity [26]. As CXCR4 plays an essential role in the localization and retention of hematopoietic stem cells in the bone marrow we must consider the safety and side effects of clinical application [20]. About that, studies show that CXCR4-deficient human T cells remain functional in a mouse model [27,28].

7 ALTERNATIVE METHODOLOGIES FOR USING CRISPR-CAS9 TO FIGHT HIV

Latent HIV-infected cells make new copies through meiosis, considering viral DNA remains integrated into the parasitized cell along with its genes. As follows, to prevent HIV-1 replication in infected T cells using genetic editing from two HIV-1 regulatory genes, *rev* and *tat*, required for viral replication and kept in different subtypes of HIV-1. Within vitro studies, the results indicate that using CRISPR-Cas9 to target HIV-1 regulatory genes may provide a fresh approach to cell protection [29].

With the CRISPR-Cas9 system, acting on the HIV-1, *tat* and *rev* regulatory genes. Those genes reach HIV-1, and have been introduced into infected patient cells since CD4+ T are cellular reservoirs in which HIV-1 may persist, even during ART.

Using the CRISPR-Cas9 gene edition to break two viral regulatory genes, *tat*, and *rev* that are indispensable for viral replication, six guide RNAs (gRNAs) were used. Three targeting the *tat* gene and three targeting the *rev* gene. To direct the Cas9 enzyme DNA cleavage towards the relevant sites in proviral DNA, the Cas9 enzyme system and gRNAs were inserted into a lentiviral vector to add the material into cultured cells [21].

Tat and *rev* expressions in latent infected CD4+ T cells after lentiviral issuance of the CRISPR-Cas9 system successfully hew the latent HIV-1 proviral genome, thereby inhibiting the reactivation of HIV-1 [21].

One of the main concerns about this subject is the potential off-target effect, which may engender important gene mutations and chromosomal translocations [30]. The reduction of off-target effects has carries the most relevant concerns in clinical application. Some researchers have proved that Cas9-mediated off-target cleavage was very limited compared to other nucleases including ZFNs, TALENS [8]. Still, a significant off-target phenomenon was detected even for sgRNAs with six or more mismatches [20].

8 CONCLUSION

The CRISPR-Cas9 system may extinguish HIV infection in promising ways, for CRISPR-Cas9 meets the establishments in the editing of CCR5 or CXCR4 co-receptors. But the repair mechanisms after cleavage aren't perfect and so are error prone. Challenges also arise in how the CRISPR-Cas9 structure is transported to cells by using various forms such as viral vectors and cell cultures.

Problems might occur when CRISPR-Cas9 acts on co-receptors. Even though side effects of permanent editing of the CCR5 gene have not been reported, some researches show that CCR5 deficiency increases the risk of symptomatic West Nile virus infection and, CXCR4 plays an essential role in the localization and retention of hematopoietic stem cells in the bone marrow. That said, we must consider the safety and side effects of clinical application as CRISPR is a new study.

Alternative methodologies for the use of CRISPR-Cas9 without involving co-receptors, make use of gene editing in latently HIV-infected cells in purpose to break two *tat* and *rev* viral regulatory genes, indispensable for viral replication. Yet, one of the main concerns is the potential off-target effect in this technique.

Therefrom, the biggest dare of CRISPR-Cas9 is to become an utterly accurate and safe technique, because as said in a recent study, there is no way to know what may happen in a long-term human being after a genetic edition with such significance. Considering the human organism a larger and a lot more complex than cell cultures or laboratory animals.

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